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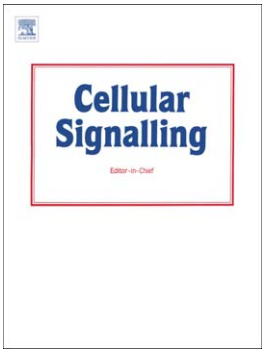

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The cyclic AMP phosphodiesterase 4D5 (PDE4D5)/Receptor for Activated C-Kinase 1 (RACK1) Signalling Complex as a Sensor of the Extracellular Nano-environment

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Abstract

The cyclic AMP and protein kinase C (PKC) signalling pathways regulate a wide range of cellular processes that require tight control, including cell proliferation and differentiation, metabolism and inflammation. The identification of a protein complex formed by receptor for activated C Kinase 1 (RACK1), a scaffold protein for protein kinase C (PKC), and the cyclic AMP-specific phosphodiesterase, PDE4D5, demonstrates a potential mechanism for crosstalk between these two signalling routes. Indeed, RACK1-bound PDE4D5 is activated by PKCα, providing a route through which the PKC pathway can control cellular cyclic AMP levels. Although RACK1 does not appear to affect the intracellular localisation of PDE4D5, it does afford structural stability, providing protection against denaturation, and increases the susceptibility of PDE4D5 to inhibition by cyclic AMP-elevating pharmaceuticals, such as rolipram. In addition, RACK1 can recruit PDE4D5 and PKC to intracellular protein complexes that control diverse cellular functions, including activated G protein-coupled receptors (GPCRs) and integrins clustered at focal adhesions. Through its ability to regulate local cyclic AMP levels in the vicinity of these multimeric receptor complexes, the RACK1/PDE4D5 signalling unit therefore has the potential to modify the quality of incoming signals from diverse extracellular cues, ranging from neurotransmitters and hormones to nanometric topology. Indeed, PDE4D5 and RACK1 have been found to form a tertiary complex with integrin-activated focal adhesion kinase (FAK), which localises to cellular focal adhesion sites. This supports PDE4D5 and RACK1 as potential regulators of cell adhesion, spreading and migration through the non-classical exchange protein activated by cyclic AMP (EPAC1)/Rap1 signalling route.

Keywords: RACK1; PDE4D5; Nanotopography; GPCRs

Highlights:

- RACK1 selectively interacts with the cyclic AMP phosphosdiesterase, PDE4D5
- RACK1 mediates protein kinase C (PKC)-dependent activation of PDE4D5
- RACK1 modulates Gs- and Gq-coupled GPCR signalling through PKC and PDE4D5
- RACK1 controls focal adhesion complex assembly and nanotopographic sensing
- RACK1 and PDE4D5 facilitates cell spreading and morphology

1. Introduction

Cyclic AMP is synthesised intracellularly following activation of transmembrane GPCRs by hormones and neurotransmitters, thereby translating extracellular ligand binding into intracellular signals by virtue of conformational changes in the receptors that traverse the cell membrane. These structural effects result in the dissociation of Gα and Gβγ heterotrimeric G-protein subunits at the plasma membrane [1]. It is the stimulatory, $G_s\alpha$, subunit that initiates the synthesis of cyclic AMP via activation of adenylate cyclase (AC) at the plasma membrane, thereby catalysing the conversion of ATP into cyclic AMP and pyrophosphate. In contrast, inhibitory $G_i\alpha$ subunits block AC activation thereby limiting cyclic AMP production. Elevated intracellular cyclic AMP levels are able to activate a select range of signalling pathways through specific interaction with effector proteins that contain cyclic nucleotide binding domains (CNBs). These include protein kinase A (PKA) [2], exchange protein activated by cyclic AMP (EPACs) [3, 4], cyclic AMP responsive ion channels (CICs) [5] and Popeye domain containing proteins Popdcs [6]. Activation of these proteins by cyclic AMP controls many aspects of cell function, including proliferation [7], differentiation [8], secretion [9], cell spreading [9, 10], inflammation [11], contractility [12] and synapse remodelling [12]. As such, cyclic AMP signalling has become an attractive target for drug development to treat of a variety of disease states.

2. Cyclic AMP Effector Proteins

For a long time PKA was thought to mediate the majority of cyclic AMP effects in most cell types, however the ability of cyclic AMP to promote activation of the small GTPase, Rap1, in the presence of PKA inhibitors prompted the search for other cyclic AMP responsive proteins. Searching *in silico* for genes containing CNBs resulted in

the discovery of the EPAC family of cyclic AMP-activated guanine nucleotide exchange factors (GEFs) [3, 4]. Unlike PKA, whose regulatory CNBs and catalytic domains are encoded by separate genes, the N-terminal regulatory and C-terminal catalytic regions of EPAC proteins are expressed within a single gene product. Furthermore, signalling from activated PKA is mediated through phosphorylation of a plethora of intracellular target proteins [13], whereas the GEF activity of EPAC proteins is principally directed towards the stimulation of Rap1 and Rap2 GTPases [3, 4]. Cyclic AMP-gated CICs are found within olfactory sensory neurons, brain, kidney and the heart. Cardiac CICs are called hyperpolarization-activated cyclic nucleotide-gated channels (HCNs) and are responsible for maintaining a regular heartbeat [5]. Similarly, Popdcs are membrane bound cyclic AMP receptors that interact with the two-pore domain potassium channel, TREK-1 [14], and Caveolin-3 [15], to regulate pacemaker activity. Indeed, deletion of Popdcs produces pronounced cardiac arrhythmia, suggesting they have a vital role in cardiac function consistent with their targeted expression within cardiomyocytes [6]. Interestingly, the cyclic AMP binding domain of Popdcs displays low sequence homology compared to other CNBs, suggesting a convergent form of evolution [14].

3. Inhibition of Cyclic AMP Signalling by the Cyclic AMP phosphodiesterase (PDE) Family

Inactivation of cyclic AMP effectors is brought about by depletion of the local cyclic AMP signal through the action of the cyclic AMP phosphodiesterase (PDE) family, which hydrolyse newly synthesised cyclic AMP to form 5'-AMP [16], and represent the major means of reducing cyclic AMP levels in cells. The phosphodiesterase family is involved in the hydrolysis of cyclic AMP and cyclic GMP, and of the 11 known classes

of cyclic nucleotide PDEs, eight are able to hydrolyse cyclic AMP [17]. Of these, the PDE4 cyclic AMP-specific family is widely expressed in a variety of tissues and accounts for the majority of cyclic AMP hydrolysis activity in cells [18]. There are four known PDE4 genes in humans, PDE4A, PDE4B, PDE4C and PDE4D, with almost complete conservation with mouse, indicating a strong evolutionary selective pressure to maintain concurrence of the genes between species [19]. Additional PDE4 isoforms are generated from alternatively spliced mRNA transcripts, based on the presence or absence of upstream conserved regions (UCRs) and unique N-terminal sequences. UCR 1 and 2 are located between the N-terminal and the catalytic site, and have important regulatory roles for the catalytic domain, where they are able to influence PDE4 activity and dimerization [20, 21]. Thus, 'short' PDE4 isoforms only have UCR2 and 'super-short' isoforms lack both UCR1 and most of the UCR2 domain [22]. UCRs appear to influence differential functions between isoforms following catalytic domain phosphorylation, with differing responses to phosphorylation by kinases, such as the MAP kinase ERK, depending on which of the UCRs are present in the individual isoform [23, 24]. Thus, long PDE4 isoforms are inhibited by ERK phosphorylation and short PDE4 isoforms are activated by ERK phosphorylation [25], with ERK phosphorylation of long PDE isoforms resulting in an increase in cyclic AMP levels and activation of cyclic AMP effectors [24]. A potential consequence of this is that PKA would become activated, leading to inhibition of ERK signalling [23, 26]. Given that ERK/MAP kinases play a key role in the control of cell growth, proliferation, differentiation and apoptosis, this would provide an intriguing negative feedback mechanism. Indeed, such a control network has been observed during the control of learning and memory [27, 28]. However, given the positive and negative regulation of PDE4 activity by ERK, the nature of the coupling between cyclic AMP and

ERK signalling pathways would be governed by the ratio between the short and long isoforms expressed in cells [25].

PDE4 isoforms are now widely accepted as important therapeutic targets. For example, PDE4 activity is the most dominant of PDE activity in inflammatory cells, and is found in eosinophils, neutrophils and CD4+ lymphocytes, suggesting an important role for PDE4 isoforms in diseases such as COPD and asthma [29]. Indeed the PDE4 inhibitors, roflumilast and cliostimast, have entered clinical trials as treatments for these disorders [29]. Of particular note are the observations that mutations in the PDE4D gene underlie increased cardiac risk and atrial fibrillation [30], osteoporosis [31] and atherosclerosis [32], which warrants further investigations into the biology of this PDE4 class. The PDE4D gene is located on chromosome 5q12 and has a complex genomic arrangement, composed of two major exon clusters, which encode the highly conserved catalytic and regulatory regions, and three exons, which encode specific N-terminals [20]. Each splice-variant isoform is characterised by a unique N-terminal domain, which is thought to be involved in intracellular targeting and protein [33-35] or lipid interactions [36]. For example, PDE4D1 and PDE4D2 are short isoforms and are largely soluble, whereas long forms PDE4D3, PDE4D4 and PDE4D5 can be located in either the cytosol or associated with cell membranes [20]. While the maximal activity (Vmax) of PDE4D3 appears to be the same in both the cytosolic and membrane fractions, those of PDE4D4 and PDE4D5 are around 3 times higher in the membrane fraction of cells [20]. The range of locations, abundance and activities of the PDE4D isoforms suggests that each splice variant has both a highly specific and unique role to play within the cell. It also appears that localisation to the particulate fraction of the cell has an influence on the PDE4D isoform enzyme properties, such as sensitivity to inhibition with PDE4-

selective drugs, such as rolipram [37], which is likely governed by the unique PDE4 N-terminal regions that determine their interactions with other cellular proteins. Notably, the phosphodiesterase PDE4D5 has been found to interact with the ubiquitously expressed WD40 signalling scaffold protein, RACK1 [34, 38]. Interaction with RACK1 appears to increase the sensitivity of PDE4D5 to the PDE4-selective inhibitor, rolipram, and facilitate its phosphorylation and activation by protein kinase C (PKC) and PKA signalling pathways [34]. Here we review our increasing understanding of this intriguing protein complex and suggest possible functions in the control of cell shape dynamics and responsiveness to the extracellular nano-environment.

4. Compartmentalisation of Cyclic AMP Signalling by Anchoring Proteins

The subcellular targeting of AC, PDE4s and associated effector proteins provides an additional layer of regulation by limiting cyclic AMP production to distinct subcellular locales. Through this compartmentalised targeting, cyclic AMP is able to activate specific subsets of effector molecules at discrete subcellular locations [39]. This form of compartmentalisation was first observed during studies on the differential effects of the Gsα-coupled receptor agonists, isoprenaline and prostacyclin, in rat cardiomyocytes. In these studies it was observed that isoprenaline, but not prostacyclin, promoted cell contractility, despite both hormones being able to stimulate elevations in intracellular cyclic AMP [40]. It was found that isoprenaline promoted cyclic AMP synthesis in both the particulate and soluble cellular fractions of cells, whereas prostacyclin only promoted cyclic AMP synthesis in the soluble fraction [40]. Thus, the subcellular location of cyclic AMP production is vitally important in determining the cellular response to elevations in intracellular cyclic AMP. Indeed, if a subcellular compartment is rich in PDEs, the cyclic

AMP signal will be limited within the local region and, conversely, if PDEs are absent, the cyclic AMP signal will be more intense and sustained [41].

It is now known that compartmentalisation of cyclic AMP signalling is orchestrated by a range of specialised anchoring proteins that interact with PDEs and cyclic AMP effector proteins to spatially control the cellular response to increases in intracellular cyclic AMP levels [41, 42]. For example, both PKA and EPAC are sequestered to distinct subcellular compartments through the actions of PKA anchoring proteins (AKAPs) [43] and EPAC anchors (e.g. RanBP2, mAKAP, ezrin and MAP1a) [44-47]. The unique targeting of anchoring proteins to subcellular structures, including cytoskeletal components or intracellular organelles, will determine which effector pathway is triggered and, therefore, the nature of the local response following cyclic AMP stimulation. Similarly, PDE4s have also been reported to be recruited to subcellular compartments through interactions with anchoring proteins, including mAKAP, β-arrestin and RACK1 [45, 48, 49]. In the case of mAKAP, the coordinated interaction of PKA, EPAC1 and ERK5 leads to a local reduction of cyclic AMP levels through stimulation of AKAP-bound PDE4D3 by PKA, and release of ERK5-mediated PDE4D3 inhibition through the actions of EPAC1, thereby providing complex regulation of cyclic AMP levels by two coordinated feedback mechanisms [45].

4.1 β-Arrestins

β-Arrestins are cytosolic proteins that mediate homologous desensitization of GPCRs by binding to agonist-occupied receptors and uncoupling them from heterotrimeric G proteins. Active GPCRs become phosphorylated by G-protein coupled receptor kinases

(GRKs) leading to the recruitment of β-arrestin to the plasma membrane resulting in the recruitment of the β-arrestin-receptor complex to clathrin coated pits [50]. In addition to the actions of GRKs, phosphorylation of receptors by either PKA or protein kinase C (PKC) directly uncouples GPCRs from their cognate G-proteins, thereby promoting signal termination. In addition, it has been shown that phosphorylation of some Gs-coupled GPCRs by PKA not only decreases their coupling to Gs but switches their coupling to Gi, resulting in a further reduction in the rate of cyclic AMP generation, through inhibition of AC, and also coupling the receptor to Gi-activated pathways, such as ERK [48]. Indeed, it has been shown that the recruitment of β-arrestin-bound PDE4D5 to active β2-adrenergic receptors controls this switch from Gs- to Gi-signalling through the degradation of local cyclic AMP levels and inhibition of PKA-mediated receptor phosphorylation [48, 49].

4.2 RACK1

RACK1 is a 36 kDa protein homologue of the β subunit of heterotrimeric G proteins (Gβ) and both are members of the large Trp-Asp40 (WD40) repeat family. The WD40 motif is highly conserved between species with the structure of RACK1 having a high degree of homology between higher mammals [51]. The function of RACK1 as a scaffold protein stems from the highly regular secondary and tertiary structure that together form an ordered ring structure made up of seven WD40 repeats. The WD40 repeats are four-stranded β-sheets with characteristic Gly-His, and Try-Asp dipeptides separated by around 40 residues. The fold is held together by ionic interactions between the conserved His-Asp residues, hydrophobic clustering and hydrogen bonding between β-strands, while the Trp determines the spacing within the fold. The sequence repeat of WD40

follows an invariant pattern whereby the β-strand N-terminal to the WD40 forms a beta sheet with the preceding fold or, in the case of the initial WD40 repeat, with the final in a head to tail conformation. This allows the WD40 repeats to come together into a cyclic structure (Figure 1). Within this ring, loops that form between the regularly folded sheets structures show the greatest solvent exposure and variability, presenting multiple protein interaction sites allowing RACK1 to bind to multiple proteins, bringing them together into functional complexes.

4.2.1 RACK1 and PKC

RACK1 was originally discovered as a binding protein for conventional PKC serine/threonine protein kinases, following their activation by tumour promoting phorbol esters or the plasma membrane component, diacylglycerol [52]. RACK1 binds activated PKC isoforms through a pseudo-substrate binding site that controls specificity of PKC-mediated signalling by translocating and anchoring the activated protein to particular cellular locations [52, 53]. As such, RACK1 is widely recognised as being an anchoring protein for the PKC family although it has subsequently been shown to bind a wide range of other cellular proteins, with a wide variety of functions [34]. The seven bladed β propeller structure of RACK1 presents a rigid ring containing multiple binding sites that allow RACK1 to act as a scaffold protein for a wide range of interacting proteins [34]. While PKCβII was the first identified binding partner, RACK1 has subsequently been shown to interact with numerous proteins, including focal adhesion kinase (FAK), Src, phospholipase Cγ, Dynamin-1, Ras-GAP, integrins and several viral proteins, including BZLF1, the EBV activation protein, the Adenovirus E1A protein and the Influenza M1 protein [34]. The recent discovery that RACK1 can form a homodimer reveals a possible mechanism for RACK1 to bind to multiple partners simultaneously [54]. RACK1 is

thought to recruit binding proteins into diverse signalling cascades by shuttling and anchoring the proteins to their appropriate subcellular locations, however the functional significance of many of these interactions remains to be established. One of the most intriguing observations, however, is that RACK1 is involved in the recruitment of PKCβII to the ribosome. A cryo-EM structure of RACK1 in complex with the human ribosome revealed various key sites for interaction close to the mRNA exit channel, with both 40S-associated proteins and ribosomal RNA (rRNA) as well as PKCβII [55-59]. Indeed, RACK1 appears to be required for efficient processing of the 18S rRNA 3'-end, which might be related to its role in translation initiation [60]. RACK1 makes contacts with three ribosomal proteins; S3, S17 and S16, with propellor blades 4 and 5 binding to the C-terminal tail of S3 and additional rRNA and S16 interactions occur with blades one and two [59]. Interestingly, the solvent exposed PKCβII binding sites within RACK1 all map to the ribosome binding face, suggesting that PKCβII binding and ribosome interaction are unlikely to occur simultaneously without significant changes in the overall structure. Nethertheless, it has been demonstrated that interaction of PKCβII and RACK1 is involved in the regulation of translation, perhaps through interactions with the ribosomal machinery [56].

4.2.2 RACK1 and GPCRs

RACK1 has also been shown to interact with the Gβγ subunit of heterotrimeric G-proteins and may therefore regulate GPCR signalling [61]. Although RACK1 appears to have little effect on general Gβγ functions, such as regulation of chemotaxis and MAPK signalling, it does appear inhibit cyclic AMP production by type 2 AC (ACII), and phosphatidylinositol hydrolysis by phospholipase Cβ2, both of which are downstream effectors of Gαs- and Gαq-coupled GPCR activation, respectively [62]. With regards to

Gαs signalling, RACK1 has been linked to the regulation of gene transcription and the actin cytoskeleton by cyclic AMP. In this regard, RACK1 has been shown to translocate to the nucleus following cyclic AMP stimulation where it induces the expression of brain-derived neurotrophic factor (BDNF) [63-66]. Intriguingly, the regulation of the BDNF gene by cyclic AMP and RACK1 appears to involve RACK1-dependent association of β-actin to the BDNF promoter [67]. The PDE4D5/RACK1 signalling complex is therefore of particular interest as it spans both the cyclic AMP and PKC signalling pathways, and may be an area of cross-talk between the two pathways in the context of GPCR signalling.

4.2.3 Interactions between PDE4D5, RACK1 and β-Arrestin

Protein-protein interaction appears to be a common feature PDE4 isoforms. For example, PDE4D3 binds to myomegalin, a large protein found in cardiac and skeletal muscle [68], PDE4A5 forms a complex with the immunophilin XAP2, an associated protein of the Hepatitis B virus X protein [69] and PDE4A4, PDE4A5 and PDE4D4 all interact with Src family tyrosine kinases [70-72]. Another, widely studied protein-protein interaction occurring between PDE4D5 and the scaffold protein RACK1 [34]. PDE4D5 is distinguishable from other PDE isoforms by 88 amino acids at the N-terminal of the enzyme, which is unique to PDE4D5 and highly conserved between mammalian species, and is responsible for recruitment of PDE4D5 to RACK1 [34]. The interaction between PDE4D5 and RACK1 was first identified by a yeast two-hybrid screen and was found to be highly specific, in that PDE4D5 does not to bind to other WD40 repeat proteins and RACK1 does not interact with other members of the PDE4 family [34]. The interaction of these two proteins is therefore both specific and unique, suggesting an important functional role for the complex. RACK1 and PDE4D5 are believed to interact in a

manner similar to the heterotrimeric G-protein subunits, Gβ and Gγ, whereby the helical segment of Gγ fits into the groove created by blades 5, 6 and 7 of the Gβ propeller (Figure 1) [38]. The C-terminal portion of the WD repeat protein is clearly important in this helix/groove-like interaction, and this has also been shown for the RACK1/PDE4D5 complex, with blades 5, 6 and 7 of RACK1 proving sufficient to interact with PDE4D5 [73, 74]. It should be noted, however, that these experiments were carried out using a RACK1 construct composed solely of WD40 repeats 5, 6, and 7 and was found to be 25% less effective at binding PDE4D5 than wild type RACK1 [74]. This suggests that the intact propeller structure of RACK1 is required for optimal binding.

The RACK1 interaction domain (RAID1) in PDE4D5 was found to consist of a segment of charged amino acids, together with a second section of hydrophobic amino acids [73]. Hydrophobic amino acids including Leu29, Val30, Leu 38, form a ridge along one side of the RAID1 helical structure (Figure 1) and Arg34 residue in the charged region was found to be essential for binding to RACK1 [73]. Subsequently, it was found that the N-terminus of PDE4D5 binds readily to both RACK1 and the GPCR regulator, β-arrestin [75]. However, it appears binding to both of these proteins cannot occur at the same time, due to overlapping binding sites within RAID1 [75]. This suggests that these proteins compete to bind to PDE4D5, with studies in HEK293 cells showing that the affinity of RACK1 binding to PDE4D5 is around double that of β-arrestin, suggesting that PDE4D5 preferentially interacts with RACK1 [75]. Selective interactions between PDE4D5 and either RACK1 or β-arrestin may underlie differing roles for PDE4D5 in cells. For example, the translocation and recruitment of the β-arrestin/PDE4D5 complex to the active β2-adrenergic receptor, contributing to the local hydrolysis of cyclic AMP and regulating the switch of receptor signalling from Gs to Gi, as described earlier [48, 49, 76, 77]. In contrast, a separate pool of

PDE4D5 appears to be constitutively associated with membrane-bound RACK1 and does not translocate response to β2-adrenergic receptor activation [37]. The RACK1/PDE4D5 complex may therefore perform different cellular functions than the β-arrestin/PDE4D5 complex. Indeed, the translocation of PDE4D5 and its cognate binding partners in response to β2-adrenergic receptor activation may depend on the cell context. For example, β-arrestin/PDE4D5 translocation seems to occur in cells that express β2-adrenergic receptors to a high level, for example in cardiomyocytes [78] or HEK293 cells stably transfected to express high levels of receptor [49, 77], and may reflect the ability of β-arrestin to develop high affinity interactions with this receptor type. The β2-adrenergic receptor may therefore present an effective recruitment site for β-arrestin/PDE4D5 signalling complexes, but not for RACK1/PDE4D5 complexes. This may depend on differences in the nature of the interaction of RACK1 and β-arrestin with PDE4D5. Accordingly, whereas RACK1 can interact with both monomeric and dimeric forms of PDE4D5, β-arrestin interacts selectively with the monomeric form, this difference may underlie the ability of β-arrestin to β2-adrenergic receptors [79]. Although RACK1 does not appear to affect translocation of PDE4D5 it does appear to act upon PDE4D5 structural stability [37]. For example, interaction with RACK1 has been found to afford PDE4D5 some protection against thermal denaturation at the plasma membrane [37]. Moreover, interaction with RACK1 at the plasma membrane also increased the affinity of PDE4D5 for cyclic AMP and rolipram, again suggesting that interaction with RACK1 can affect the conformation of PDE4D5, perhaps priming it for activation in the particulate fraction of cells [37]. In this regard, the implication that RACK1 binding to PDE4D5 lowers the effective concentration of rolipram required for inhibition of PDE activity, suggests that future pharmaceuticals based on synthetic RACK1 analogues in combination with lower doses of rolipram could potentially be used to produce biological effects with potentially lessened side-effects.

Another potential role of the PDE4D5/RACK1 signalling complex is to form multi-protein complexes. Indeed, given that RACK1 was originally identified by its ability to bind activated conventional PKC isoforms, the RACK1/PDE4D5 complex forms a point of crosstalk between the cyclic AMP and PKC signalling pathways. We found that PKCα and RACK1 are capable if interacting at the plasma membrane in HEK293 cells that had been stimulated with the PKC activator, phorbol 12-myristate 13-acetate, which causes PKC activation and membrane recruitment [37]. We found that this coincided with activation of PDE4D5 in the same cell fraction [37]. Furthermore, comparison of RACK1-bound PDE4D5 with a non-RACK1 binding mutant demonstrated that interaction with RACK1 is essential for the activation of PDE4D5 by PKCα [37]. The complex formed between PDE4D5 and RACK1 may therefore provide a point of crosstalk between the cyclic AMP and PKC signalling pathways as well as a potential point of negative feedback. In this regard, it has been shown that, under certain circumstances, increased cyclic AMP levels have been shown to activate PKC [80], and could therefore lead to signal termination through the activation of PKC-regulated PDEs, such as PDE4D5. This type of feedback control could perhaps underlie complex biological phenomena, such as the control of the mammalian circadian clock, which involves both cyclic AMP signalling through EPAC1 and the PKCα isoform in complex with RACK1 [81, 82].

5. PDE4D5/RACK1, Nanotopographic Sensing, Wound Healing and Cancer

RACK1 has been shown to be important for linking growth factor receptor activation to the integrin-mediated promotion of cell migration [83, 84]. Integrins are αβ heterodimeric

receptors that act as sensors for cues like extracellular matrix and nanometric topology, which promote integrin clustering at interaction points called focal adhesions [85]. RACK1 has been shown to interact with integrin β-chains, which appears to be mediated by WD repeats 5 to 7 of RACK1 [86], and localise to focal contacts in order to organise focal adhesions, leading to increased cell adhesion and focal adhesion size [48-53]. These effects are due to the ability of RACK1 to interact with and coordinate key intracellular signalling molecules, such as PKCε, Src, and ERK at focal adhesion sites (Figure 2). For example, RACK1 directly links PKCε to integrin β-chains [87]. Disruption of the PKCε targeting to integrin receptors, by knockdown of RACK1 or over-expression of a truncated form of RACK1 that lacks WD repeats 5 to 7, leads to impaired adhesion and migration of cells [87]. Src is a tyrosine kinase that is involved in phosphorylating specific focal adhesion proteins, including the integrin-regulated, non-receptor tyrosine kinase focal adhesion kinase (FAK) [88-91]. FAK is activated and localized at focal adhesions in cells adhered to extracellular matrices and is primarily recruited to sites of integrin clustering via interactions with integrin-associated proteins, such as talin and paxillin [92]. Overexpression of RACK1 in fibroblasts increases phosphorylation of FAK concomitant with an increase in the number of focal adhesions and stress fibre formation [93, 94]. This relationship may not occur in every cell type however since, paradoxically, silencing of RACK1 expression in REF52 cells was also found to lead to enhanced focal adhesion formation and decreased cell motility [95]. The down-stream target of FAK is the MAP kinase, ERK, which is anchored at newly formed focal adhesions by RACK1 and is responsible for controlling focal adhesion organisation following integrin engagement [95, 96]. Indeed, RACK1 is required for ERK activation in response to cell adhesion but not from classical growth factor activation [95]. Furthermore, RACK1 also associates with the other core kinases of the ERK pathway, Raf and MEK [95]. This

indicates that RACK1 integrates signals from upstream activation by integrins to downstream targets at focal adhesions.

5.1 RACK1 and Nanotopography

One of the functions of RACK1 therefore may be to control the interactions of signalling pathways involved in the coordination of cell adhesion, movement, division and wound healing. To explore this we investigated the role of RACK1 in regulating the response of MCF-7 human breast cancer cells to contact guidance on custom-engineered nanometrically deep grooves [93]. The process of cell contact guidance is essential in vivo for every physiological process involving cell migration. For example, contact guidance has been implied in various morphogenetic movements including neural crest cell migration, primordial germ cell migration and guidance of axonal growth cone and is also implicated as an important component of several homeostatic processes, such as wound healing and tissue repair [97]. Specialised surface topographies like grooves influence cell responses at many levels, from initial attachment and migration to differentiation and generation of new tissue [98]. We therefore used photolithography to produce regular grooves with μm scale pitch and nm scale depth. We found that enforced expression of RACK1 limited the response of cells to contact guidance on nanometric grooves whereas ablation of RACK1 protein, with specific anti-sense oligonucleotides, led to a dramatic enhancement of bi-directional extension of cells on the nanometrically-deep grooved surfaces, with a corresponding loss of focal adhesions and stress fibres [93]. RACK1 therefore exerts a tonic inhibitory effect on cell contact guidance, while positively promoting an adhesive phenotype. Together the data presented in this study defines a role for RACK1 as a positive regulator of cell adhesion, yet a negative regulator of cell contact guidance on nanometrically-deep grooves. These observations are rather

counter-intuitive and suggest that formation of focal adhesion complexes and the bi-directional movement of cells during contact guidance are mutually exclusive processes that may actually antagonise one another. One potential mechanism by which RACK1 limits contact guidance is through the regulation of the activity of FAK, as described above. Given that PDE4D5 and RACK1 have been found to form a tertiary complex with FAK that localises at cellular focal adhesion sites [99, 100], this places PDE4D5 and RACK1 as potential regulators of cell adhesion, spreading and migration and, potentially, as sensors of the extracellular nano-environment.

5.2 RACK1/PDE4D5 and Cancer

There is a long history linking elevations in cyclic AMP to the regulation of cell adhesion and spreading and it has recently become known that these effects are mediated by the cyclic AMP sensor protein, EPAC1, which activates the small G protein, Rap1, promoting cell adhesion, and cytoskeletal reorganisation [11, 101]. EPAC1/Rap1 signalling controls a range of adhesive and cohesive pathways connected by the dynamic cytoskeleton [102, 103] and, in many cases, these pro-adhesive actions of EPAC1 synergise with PKA [10, 104, 105]. Rap1 is a member of the Ras family, but is largely involved in the control of cell morphology, adhesion and cohesion [106] functions that underlie the ability of EPAC1 to regulate cell adhesion [107], cell-cell contact stability within the vascular endothelium [108], as well as cell spreading [109]. Rap1 cycles between an inactive, GDP-bound form and the active GTP-bound conformation. The cycling of activation status is regulated by the stimulatory effects of GEFs which induce GTP binding, or GTPase activating proteins (GAPs) which promote GTP hydrolysis and the GDP-bound state [106]. The morphological changes that active Rap1 promote include uniform, isotropic membrane projections resulting in large increases in cell area.

Interestingly, this response appears to be independent of classical cyclic AMP mediated focal adhesion and integrin stabilisation [110-112], but rather cytoskeletal reorganisation occurs through Rap1-mediated inhibition of the Rho family of small GTPases [105, 113-115]. How the inhibition of Rho GTPases by Rap1 is brought about is not yet known, however it might involve RACK1 promoted, adhesion-mediated activation of ERK2 that that regulates the peripheral localization of the Rho-inhibitor enzyme, p190A-RhoGAP [96]. Recently, the actin- cytoskeletal linker protein, ezrin, was also found to be required for EPAC1-mediated cell spreading [10, 109], however the manner in which cyclic AMP is able to regulate ezrin to produce cell spreading is currently unknown. Given the link between cyclic AMP signalling and the regulation of cell morphology, it is tempting to speculate that the PDE4D5/RACK1 complex plays a key role in these mechanisms. This is borne out by two studies from the Frame laboratory. Firstly, Serrels et al describe a complex formed between FAK, RACK1 and PDE4D5 that is recruited to focal adhesions and promotes cell polarity [100]. This may be important because RACK1 is expressed in both normal and malignant cells, while FAK and PDE4D5 are both elevated in the cancer cells. Indeed, introducing a mutant FAK, that lacks the ability to interact with RACK1, into cancer cells where endogenous FAK had been deleted led to FAK being no longer promote the formation of nascent actin adhesion structures in spreading cells, leading to impaired directional responses, including wound-induced polarization and chemotactic migration. Moreover, in a separate study, the same group demonstrated that cyclic AMP signalling through EPAC suppresses polarization of squamous cancer cells and that the RACK1-binding mutant of FAK induces activation of the EPAC-effector, Rap1, which was linked to impaired cell polarization [99]. Together, these two studies suggest that the integrin-linked FAK/RACK1/PDE4D5 signalling complex suppresses Rap1 at appropriate times, in a spatially regulated manner, as cells first sense their environment

and then make decisions about nascent adhesion stabilization and polarization. This suggests that signalling through FAK/RACK1/PDE4D5/Rap1 may contribute to tumour development.

6. Conclusions

Overall it appears that RACK1 has the potential of physically recruiting PDE4D5 to regulate a range on key intracellular signalling process, including GPCR and integrin signalling through PKA, EPAC, and other effector routes. Further work is required to link these phenomena to key biological events, but initial studies imply that this may include central cell biology processes, including cell morphology, spreading and interaction with the extracellular nano-environment.

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Figure Legends

Figure 1 Hypothetical prediction of the PDE4D5/RACK1 interface. The Gβ/γ subunit complex (3SN6) was aligned to human RACK1 (4AOW). Several hydrophobic residues of Ggamma cluster within the groove between blades 6 and 7 of Gβ. Assuming a similar mode of binding, the peptide structure of the RAID of PDE4D5 (1E9K) was overlaid onto Gγ, with the hydrophobic binding regions identified as key for RACK1 (L29, V30, L33) aligned to hydrophobic residues that underlie Gβ/γ.

Figure 2 Mechanisms Linking Integrin Activation at Focal Adhesion Complexes (FAC). RACK1 coordinates the interaction of key intracellular signalling molecules at FACs following αβ integrin engagement by the extracellular matrix (ECM). FACs are also composed of cytoskeletal proteins, including talin, actin, vinculin and paxillin. Src is one of the central kinases involved in phosphorylating specific focal adhesion proteins on tyrosine residues (Y), including the focal adhesion kinase, FAK. RACK1 also increases phosphorylation of FAK concomitant with an increase in the number of focal adhesions and actin stress fibre formation. The mechanisms linking FAK activation to focal adhesion formation is known to be through ERK.

Figure 1

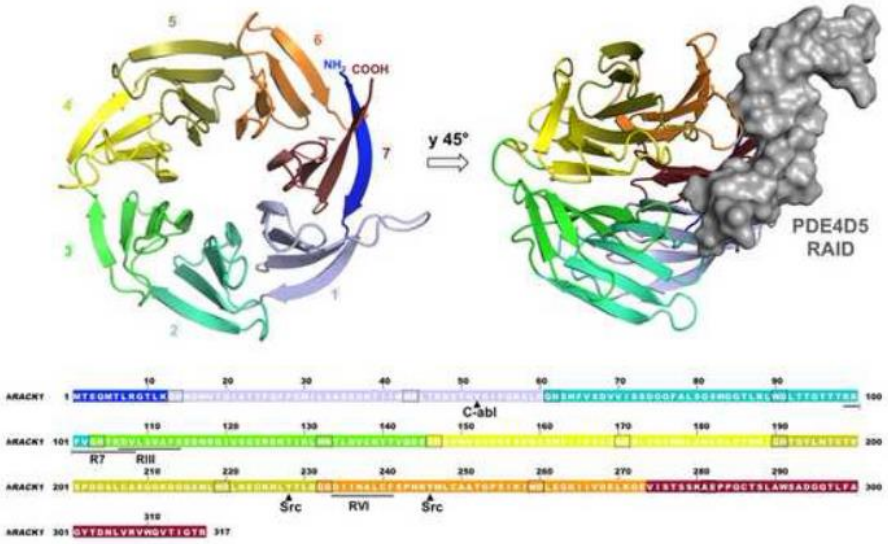

Figure 2